ORIGINAL ARTICLE

Chronic Thalidomide Administration Enhances Vascular Responsiveness to Vasopressin in Portal-systemic Collaterals of Bile Duct-ligated Rats

Ching-Chih Chang[1,4], Sun-Sang Wang[3,4], Hui-Chun Huang[2,4], Fa-Yauh Lee[1,2,4]*, Han-Chieh Lin[2,4], Jing-Yi Lee[1], Yi-Chou Chen[1], Shou-Dong Lee[2,4]

Divisions of [1]General Medicine and [2]Gastroenterology, Department of Medicine, Taipei Veterans General Hospital, [3]Taipei Municipal Gan-Dau Hospital, and [4]National Yang-Ming University School of Medicine, Taipei, Taiwan, R.O.C.

Background: Arginine vasopressin (AVP) controls gastroesophageal variceal bleeding, partly due to its vasoconstrictive effect on portal-systemic collaterals. It has been shown that chronic thalidomide treatment decreases portal pressure, attenuates hyperdynamic circulation and inhibits vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF)-α in partially portal vein-ligated rats. This study investigated the effects of chronic thalidomide treatment on portal-systemic collateral vascular responsiveness to AVP in common bile duct-ligated (CBDL) cirrhotic rats.

Methods: In the first series, CBDL-induced cirrhotic rats received thalidomide (50 mg/kg/day orally) or distilled water (control) from the 35th to 42nd day after ligation. On the 43rd day after ligation, the body weight, mean arterial pressure, portal pressure, and heart rate were measured. An *in situ* collateral vascular perfusion model was used to obtain the cumulative concentration–response curves of collateral vessels to AVP (10^{-10} to 3×10^{-7} M). Plasma levels of VEGF and TNF-α were measured, and expressions of VEGF and TNF-α mRNA in the left adrenal veins were also determined. In the second series, the cumulative concentration–response curves of collateral vessels to AVP in CBDL rats with or without thalidomide (10^{-5} M) preincubation in the perfusate were obtained.

Results: The thalidomide and control groups were not significantly different in terms of heart rate, mean arterial pressure and portal pressure ($p > 0.05$). The collateral vascular perfusion pressure change to AVP was significantly enhanced at 10^{-8} M after thalidomide treatment ($p = 0.041$). Compared with the control group, thalidomide-treated rats had significantly lower plasma VEGF levels ($p < 0.001$), accompanied by an insignificant reduction in plasma TNF-α levels ($p > 0.05$). The expressions of VEGF and TNF-α mRNA in the left adrenal veins of thalidomide-treated CBDL rats were not significantly changed compared with those of the control group. In addition, thalidomide did not significantly elicit changes in vascular responsiveness to AVP in collateral vessels of CBDL rats when it was added into the perfusate.

Conclusion: In cirrhotic rats, chronic thalidomide treatment improves the portal-systemic collateral vascular responsiveness to AVP, which was partly related to VEGF inhibition. [*J Chin Med Assoc* 2009;72(5):234–242]

Key Words: portal-systemic collaterals, thalidomide, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), vasopressin

Introduction

Acute gastroesophageal variceal bleeding is a severe complication in patients with liver cirrhosis and portal hypertension.[1] Arginine vasopressin (AVP) has been used to treat esophageal variceal bleeding for decades, and clinical trials revealed that about 50% of the bleeding episodes could be controlled,[2–4] though the vascular hyporesponsiveness to AVP during acute hemorrhage compromises its hemostatic effect.[5,6] AVP is a strong vasoconstrictor capable of inducing splanchnic vasoconstriction, followed by decreased portal venous blood flow and portal pressure.[7] In addition, lower azygos venous blood flow and intravariceal pressure in cirrhotic patients were found after AVP administration,[8,9] suggesting an effect of AVP on portal-systemic

*Correspondence to: Dr Fa-Yauh Lee, Division of General Medicine, Department of Medicine, Taipei Veterans General Hospital, 201, Section 2, Shih-Pai Road, Taipei 112, Taiwan, R.O.C.
E-mail: fylee@vghtpe.gov.tw • Received: December 25, 2008 • Accepted: April 8, 2009

collaterals. In our previous studies, a direct vasoconstrictive effect of AVP was demonstrated on the portal-systemic collaterals of portal-hypertensive and cirrhotic rats.[10,11] Therefore, AVP-induced collateral vasoconstriction may further contribute to the control of variceal hemorrhage.

Thalidomide (α-N-phthalimidoglutarimide), a derivative of glutamic acid, has been incriminated for its teratogenic effect in the past.[12] Recently, it was reported to be helpful in patients with diverse immunologic or inflammatory disorders.[13,14] It suppresses tumor necrosis factor (TNF)-α production by accelerating the degradation of TNF-α mRNA and vascular endothelial growth factor (VEGF) related angiogenesis.[13–16] In the portal hypertensive state, chronic administration of thalidomide reduces TNF-α and nitric oxide (NO) production as well as ameliorates the portal pressure and hyperdynamic circulation in partially portal vein-ligated (PVL) rats.[17] However, whether or not thalidomide enhances collateral vascular responsiveness to AVP is unknown.

In this study, the effects of acute and chronic thalidomide administration on AVP-induced vasoconstriction were investigated in cirrhotic rats. The plasma levels and expressions of VEGF and TNF-α mRNA in the collateral vessels were also evaluated.

Methods

Animal model

Male Sprague-Dawley rats weighing 240–270 g at the time of surgery were used for experiments. The rats were housed in a plastic cage and allowed free access to food and water. All rats were fasted for 12 hours before the operation. Rats with secondary biliary cirrhosis were induced by common bile duct ligation with formalin infusion (CBDL).[18] Under ketamine anesthesia (100 mg/kg, intramuscularly), the common bile duct was exposed through a midline abdominal incision. A PE-10 catheter was used to catheterize the common bile duct, and the duct was then doubly ligated with 3-0 silk. The first ligature was made below the junction of the hepatic ducts and the second ligature above the entrance of the pancreatic duct. Then, 10% formalin (~100 μL/100 g) was slowly injected in the biliary tree to prevent the subsequent dilatation of the ligated residual bile duct. The PE-10 catheter was removed and the ligatures tightened, followed by section of the common bile duct between the ligatures. The incision was then closed and the animal allowed to recover. According to the literature, a high yield of secondary biliary cirrhosis was noted 5 weeks after the ligation.[18–20] To avoid coagulation defects, CBDL rats received weekly vitamin K injection (50 μg/kg intramuscularly). The perfusion studies were performed in overnight-fasted rats 6 weeks after the operation under anesthesia by ketamine (100 mg/kg body weight intramuscularly). The body weights of rats were measured on the day of perfusion studies. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 86-23, revised 1985). This study was approved by Taipei Veterans General Hospital's Animal Committee.

Experimental design

Two groups of CBDL rats received either thalidomide (50 mg/kg/day orally; kindly provided by TTY Biopharm Co., Ltd., Taipei, Taiwan) or distilled water (control) from the 35th to 42nd day after ligation. On the 43rd day after ligation, rats were anesthesized with ketamine hydrochloride (100 mg/kg, intramuscularly) and the body weight, mean arterial pressure, portal pressure, and heart rate were measured.

Two series of experiments with the *in situ* collateral vascular perfusion model were performed. In the first series (thalidomide group, $n=10$; control group, $n=10$), cumulative concentration-response curves of collateral vessels were determined by graded final concentrations of AVP in escalation with a constant flow rate (12 mL/min). The final concentrations in the perfusate were from 10^{-10} M to 3×10^{-7} M of AVP. Each new concentration was allowed to stabilize for 3 minutes before the next higher concentration was added. The plasma levels of VEGF and TNF-α were determined in the thalidomide-treated and control groups. In addition, the left adrenal veins and the major collateral vessels of cirrhotic rats were obtained for testing mRNA expression of VEGF and TNF-α. In the second series, 10^{-5} M thalidomide or vehicle (Krebs solution) was added into the perfusate of collateral perfusion study (thalidomide group, $n=6$; control group, $n=7$) in CBDL cirrhotic rats to evaluate the direct vascular effect of thalidomide in cirrhotic rats. The cumulative concentration-response curves of both groups were obtained.

Systemic and portal hemodynamic measurements

The right internal carotid artery was cannulated with a polyethylene PE-50 catheter connected to a Spectramed DTX transducer, and permanent recordings of mean arterial pressure was made on a multichannel

recorder (model RS 3400; Gould Inc., Cupertino, CA, USA). The heart rate was calculated from the recordings. The abdominal cavity was entered through a midline incision, and the distal mesenteric vein was exposed and Cannulated with a saline-filled 18-gauge Teflon cannula and fixed with cyanoacrylate glue. This cannula was connected with a PE-50 tubing to a Spectramed DTX transducer. The abdomen was then closed and the portal pressure recorded on a multichannel recorder.[21]

In situ perfusion preparation for portal-systemic collaterals

The *in situ* perfusion system was performed as described in previous reports.[10,11,22] Both jugular veins were cannulated with 16-gauge Teflon cannulas to ensure an adequate outflow without any resistance even at high flow rates. Heparin (200 μ/100 g body weight) was injected through one of the cannulas. The abdomen was then opened and an 18-gauge Teflon cannula inserted in the distal mesenteric vein was used as the perfusate inlet. To exclude the liver from perfusion, the second loose ligature around the portal vein was tied. The animal was transferred into a warm chamber (37 ± 0.5°C). The temperature around the perfusion area was continuously monitored with a thermometer placed inside the mesentery and was maintained at approximately 37 ± 0.5°C with a thermostatic pad and temperature-controlled infrared lamp. An open-circuit perfusion was then started with Krebs solution (composition in mM: NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; dextrose 11.0; pH 7.4; 37 ± 0.5°C) via the mesenteric cannula by a roller pump (model 505S; Watson-Marlow Ltd., Falmouth, Cornwall, UK). The perfusate was equilibrated with carbogen gas (95% O_2–5% CO_2) by a silastic membrane lung.[23] Both the jugular vein cannulas were simultaneously opened to allow a complete washout of the blood. Pneumothorax was created by opening slits through the diaphragm to increase resistance in pulmonary arteries and to prevent the perfusate from entering the left heart chambers. The portal-systemic collaterals were then perfused with oxygenated (95% O_2–5% CO_2) Krebs solution containing 3% wt/vol albumin (factor V bovine serum albumin; Sigma, St Louis, MO, USA). The effluent of the perfusion was collected in a reservoir and was not recirculated. To continuously monitor and record the pressure in the portal-systemic collaterals, a Spectramed DTX transducer attached to the Gould model RS 3400 recorder was connected to a side arm placed just proximal to the perfusion cannula, with the zero placed at the level of the right atrium. Because the temperature and pressure of the system stabilized within 20 minutes, all the experiments were performed 25 minutes after starting perfusion at a constant rate of 12 mL/min.[11] After the stabilization period, the perfusion flow rate was kept constant (12 mL/min) in determining the cumulative concentration-response curves.

Cumulative concentration-response curve study

In the series one study, the cumulative concentration-response curves of collateral vessels were determined by using final concentrations of 10^{-10}, 10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8}, 10^{-7} and 3×10^{-7} M of vasopressin in the perfusate. The final concentrations were successfully reached by carefully adding calculated amounts of vasopressin. Each new concentration was allowed to stabilize for 3 minutes before the next higher concentration was added. Only 1 concentration-response curve was performed in each preparation. After testing experimental agents, the contracting capability of the collateral vessels was challenged with a 125 mM KCl solution at the end of the experiments. In the series two study, the collateral vessels were preincubated with thalidomide (10^{-5} M) to test its direct effect on the collateral responsiveness to AVP. The cumulative concentration-response curves of collateral vessels were also determined by using final concentrations of 10^{-10}, 10^{-9}, 3×10^{-9}, 10^{-8}, 3×10^{-8} and 10^{-7} M of vasopressin in the perfusate.

Determination of plasma TNF-α levels

The plasma samples were centrifuged at 3,000 rpm for 10 minutes at 4°C and stored at –80°C until tested. Plasma TNF-α levels were measured with a commercially available solid-phase sandwich enzyme-linked immunosorbent assay (rat TNF-α kits; R&D Systems Inc., Minneapolis, MN, USA) according to the protocol supplied by the manufacturer. The standards and samples were incubated with a TNF-α antibody-coated 96-well microtiter plate. The wells were washed with buffer and then incubated with anti-TNF-α antibody conjugated to horseradish peroxidase for 2 hours. This was washed away, and color was developed in the presence of tetramethyl benzidine chromogen substrate. The intensity of the color was measured in a Biokinetics Reader (Bio-Tek Instruments Inc., Winooski, VT, USA) by reading the absorbance at 450 nm with a correction wavelength of 570 nm. We compared the samples against the standard curve to determine the amount of TNF-α present. All samples were run in duplicate. The lower limit of sensitivity for TNF-α by this assay was 5 pg/mL. The intraassay and interassay coefficients of variation were 5.1% and 9.7%, respectively.

Determination of plasma VEGF levels

The plasma levels of VEGF were measured using commercially available enzyme-linked immunoabsorbent assay kits (R&D Systems Inc.) according to the manufacturer's instructions. Briefly, standards and samples were pipetted into the wells containing a monoclonal antibody specific for VEGF. After washing, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Then, a substrate solution was added to the wells and the color development in proportion to the amount of VEGF bound was stopped with a stop solution. Standard curves were constructed using serial dilutions of recombinant VEGF. The intensity of the color was measured to the absorbance of 450–600 nm with a Bio-kinetics Reader (Bio-Tek Instruments Inc.). The intra- and interassay variations of these assays were less than 10%.

Expressions of VEGF and TNF-α mRNA in the left adrenal veins of CBDL rats

The left adrenal veins of chronic thalidomide or distilled water-treated cirrhotic rats were obtained. Total RNA was extracted from the vessel with an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.[24,25] Reverse transcription of 0.2 μg of RNA using ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA) was carried out in a total volume of 20 μL according to the following protocol: 4 μL of RNA (0.2 μg) and 1 μL of oligo dT (0.5 μg) were incubated at 70°C for 5 minutes and then chilled immediately at 4°C for 5 minutes. The reacting mixture was incubated with 15 μL of reverse transcriptase mix (RNase-free water 6 μL, ImProm-II™ 5X reaction buffer 4 μL, $MgCl_2$ 3 μL, dNTP mix (10 μM each dNTP) 1 μL, ImProm-II™ reverse transcriptase 1 μL) at 42°C for 60 minutes. The resulting cDNA samples were frozen at this stage until polymerase chain reaction (PCR).

PCR was performed using primers specific to VEGF isoform (forward 5′-CCT CCG AAA CCA TGA ACT TTC TGC TC-3′ and reverse 5′-CAG CCT GGC TCA CCG CCT TGG CTT-3′; annealing temperature 60°C) and TNF-α (forward 5′-CTC AAA GAC AAC CAA CTG GTG GTA-3′ and reverse 5′-ACA GAG CAA TGA CTC CAA AGT AGA CC-3′; annealing temperature 62°C). A constitutively expressed gene, β-actin, was used as internal control, and the primers for β-actin were 5′-TTGTAACCAACTGGGACGATATGG-3′ (sense) and 5′-GATCTTGATCTTCATGGTGCTAGG-3′ (antisense), respectively. The primers for VEGF, TNF-α and β-actin were designed to allow amplification of 593, 343 and 764 base-pair fragments, respectively. A negative control of the reaction was included in each set of the experiments. The tubes were placed in the thermocycler (Biometra® T Gradient Thermocycler; Biometra GmbH, Göttingen, Germany) at 95°C for 5 minutes for initial denaturation, followed by 35 cycles of the following sequential steps: 30 seconds at 94°C (denaturation) and 45 seconds at 72°C (extension). The final extension was performed at 72°C for 10 minutes: 10 μL of the PCR-amplified mixture was subjected to electrophoresis on a 2% agarose gel, and cDNA was visualized by ethidium bromide staining. Location of the predicted PCR products (base pairs) was confirmed by using a 100-base-pair ladder (Gibco BRL, Gaithersburg, MD, USA) as a standard size marker. The gel was then photographed, and the PCR products were quantified by a digitalizing software (Kodak Digital Science™ ID Image Analysis Software, Eastman Kodak Comp., Rochester, NY, USA). The intensities of the VEGF and TNF-α signals were standardized against that of β-actin from the same RNA sample and expressed as VEGF/β-actin and TNF-α/β-actin ratio for comparison.

Drugs

Thalidomide was kindly provided by TTY Biopharm Co., Ltd., Taipei, Taiwan. AVP and the reagents for preparing Krebs solution were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA). All the solutions were freshly prepared on the days of experiment.

Data analysis

All results are expressed as mean ± standard error of the mean. The changes in perfusion pressure (mmHg) over baseline were calculated for each concentration in each preparation. The concentration of vasopressin exhibiting 50% of the maximal response (EC50) in each preparation was calculated from sigmoid logistic curves and expressed as negative log molar (–log M). Statistical analyses were performed using an unpaired Student's *t* test. Results were considered statistically significant at a 2-tailed *p* value of less than 0.05.

Results

Hemodynamic effects and plasma VEGF and TNF-α levels after thalidomide treatment

Table 1 shows the body weights, baseline hemodynamic parameters, plasma VEGF and TNF-α levels of the chronic thalidomide and distilled water-treated (control) CBDL rats. The mean arterial pressure, heart rates, portal pressure and baseline perfusion pressure were similar between the 2 groups. The plasma levels of VEGF were significantly decreased after thalidomide

Table 1. Body weights, plasma levels of TNF-α, VEGF and hemodynamic parameters in chronic thalidomide-treated and control common bile duct-ligated rats

	Thalidomide ($n=10$)	Control ($n=10$)
Body weight (g)	359 ± 12	379 ± 15
MAP (mmHg)	99 ± 4	97 ± 6
HR (beats/min)	288 ± 17	292 ± 17
PP (mmHg)	16.8 ± 0.7	17.6 ± 1.1
Baseline PP (mmHg)	16.9 ± 0.8	17.7 ± 1.0
TNF-α (pg/mL)	16.4 ± 1.9	20.1 ± 4.0
VEGF (pg/mL)	5.0 ± 1.4	15.5 ± 2.1*

$p < 0.001$. TNF-α = tumor necrosis factor-α; VEGF = vascular endothelial growth factor; MAP = mean arterial pressure; HR = heart rate; PP = portal pressure; baseline PP = baseline perfusion pressure.

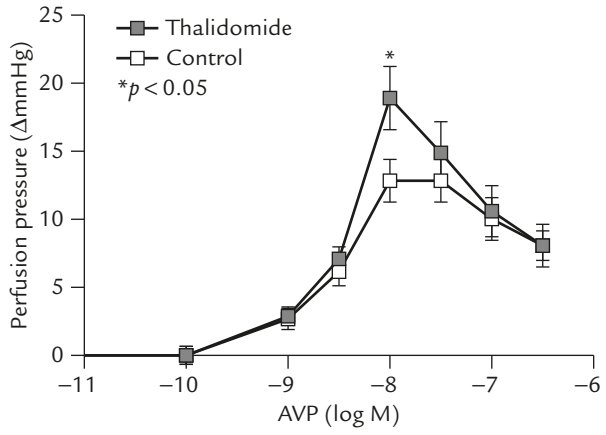

Figure 1. Concentration-response curves to arginine vasopressin (AVP) in portal-systemic collateral vascular beds of thalidomide and distilled-water (control)-treated common bile duct-ligated rats.

Table 2. Body weights and hemodynamic parameters in common bile duct-ligated rats with or without thalidomide preincubation in perfusion study

	Thalidomide ($n=6$)	Control ($n=7$)
Body weight (g)	325 ± 32	318 ± 10
MAP (mmHg)	92 ± 9	101 ± 4
HR (beats/min)	259 ± 21	293 ± 12
PP (mmHg)	17.2 ± 2.1	17.6 ± 1.3
Baseline PP (mmHg)	18.1 ± 1.7	18.0 ± 1.4

MAP = mean arterial pressure; HR = heart rate; PP = portal pressure; baseline PP = baseline perfusion pressure.

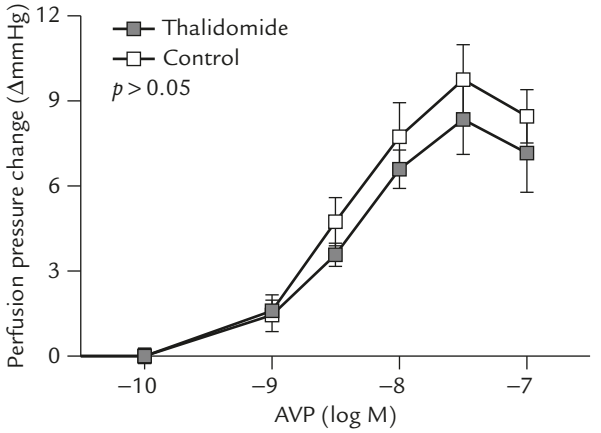

Figure 2. Concentration-response curves to arginine vasopressin (AVP) in portal-systemic collateral vascular beds of common bile duct-ligated rats preincubated with thalidomide or vehicle (Krebs solution).

treatment (thalidomide *vs.* control: 5.0 ± 1.4 pg/mL *vs.* 15.5 ± 2.1 pg/mL; $p < 0.001$). There was also a trend of lower plasma TNF-α levels in the thalidomide-treated group. It, however, did not reach statistical significance (thalidomide *vs.* control: 16.4 ± 1.9 pg/mL *vs.* 20.1 ± 4.0 pg/mL; $p = 0.416$).

Collateral vascular responsiveness to AVP in chronic thalidomide and distilled water-treated CBDL rats

Figure 1 depicts the concentration-response curves obtained at a constant perfusion flow rate by the cumulative addition of AVP into the perfusate for the 2 groups. The maximal perfusion pressure change occurred at the concentration of 10^{-8} M, and that of the thalidomide group was significantly higher compared with that of the control group (18.9 ± 2.4 mmHg *vs.* 12.8 ± 1.3 mmHg; $p = 0.041$). There was no significant difference in EC50 between the 2 groups (thalidomide *vs.* control (log-EC50): –7.69 ± 1.44 *vs.* –8.90 ± 0.18; $p = 0.470$).

Collateral vascular response to AVP with or without thalidomide preincubation in CBDL rats

Table 2 shows the baseline hemodynamic parameters and body weights of the 2 groups of CBDL rats with or without thalidomide preincubation in the perfusate. The body weights, mean arterial pressures, heart rates, portal pressure and baseline perfusion pressures were similar between these 2 groups. Figure 2 shows no significant perfusion pressure changes at different concentrations of these 2 groups ($p > 0.05$).

Expressions of VEGF and TNF-α mRNA in the left adrenal veins of thalidomide-treated and control CBDL rats

The mRNA expressions of VEGF were not significantly decreased in the left adrenal veins of the thalidomide-treated group as compared with the control group (VEGF/β-actin of thalidomide group *vs.* control group: 0.39 ± 0.05 *vs.* 0.42 ± 0.05; $p = 0.65$) (Figure 3).

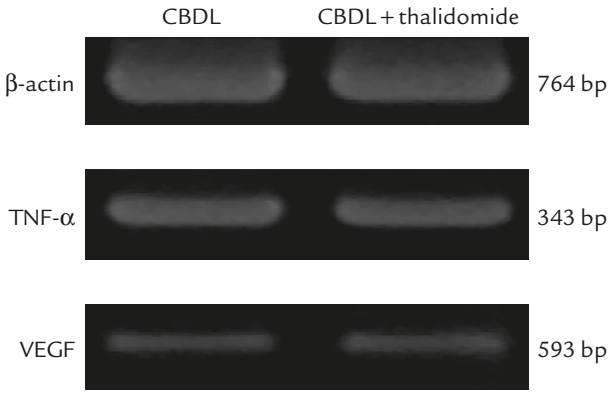

Figure 3. Representative diagram of vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF)-α mRNA expressions in the left adrenal veins of common bile duct-ligated (CBDL) rats after chronic thalidomide and distilled water treatment.

There were also no significant changes in TNF-α mRNA expression (TNF-α/β-actin: 1.15 ± 0.18 *vs.* 1.27 ± 0.19; $p = 0.66$) in the left adrenal veins between the 2 groups (Figure 3).

Discussion

In a previous study, Lopez-Talavera et al found that thalidomide elevated mean arterial pressure and decreased portal pressure in partially PVL rats.[17] The alleviated hemodynamic derangements were ascribed to TNF-α synthesis inhibition by thalidomide. However, in our study, mean arterial pressure and heart rate were not significantly different between the thalidomide-treated and control groups in CBDL-induced cirrhotic rats. The plasma TNF-α levels were also decreased after thalidomide treatment in our study but did not reach statistical significance as compared with the control group. Thalidomide has been found to inhibit TNF-α through enhancing its mRNA degradation and has a marked regulatory influence on the TNF-α protein.[15,26] Recently, it has been reported that multiple pathways are involved in the thalidomide action, including inhibition of nuclear κB activity through suppression of IκB kinase activity and suppression of TNF-α-dependent Kupffer cell sensitization.[27,28] Elevated blood levels of TNF-α have been demonstrated in patients and animals with chronic liver disease and cirrhosis.[29,30] TNF-α participates in the development of the hyperdynamic circulatory state of portal hypertension mainly through an increased release of NO.[17,31,32] In addition, the decreased vascular tone and vasodilation by TNF-α may also be mediated via prostacyclin,[32] calcitonin-gene related peptide[33] or activation of potassium channels in smooth muscle.[34] Nevertheless, in another study of 20 abstinent patients with stable alcoholic cirrhosis and esophageal varices,[35] oral thalidomide 200 mg/day for 2 weeks did not alter systemic hemodynamic parameters and plasma levels of TNF-α. In fact, in *ex vivo* experiments, individuals with portal hypertension treated by thalidomide showed potent inhibition of both basal and lipopolysaccharide-stimulated TNF-α production in peripheral blood monocytes in the absence of plasma TNF-α level changes.[35] Since plasma levels of TNF-α show wide variability with studied animals and are influenced by various hepatic injuries caused by CBDL, they may not be a reliable marker to test the efficacy of the inhibitory activity of thalidomide. Though the actual reason for discrepant results between these studies is unknown, different experimental models and dosages may also play important roles.

In the present study, portal pressure was not altered by thalidomide treatment. In the study led by Muñoz in portal-hypertensive rats,[32] portal pressure was unchanged after short- and long-term TNF-α inhibition, which is in contrast to the results of Lopez-Talavera et al's study.[31] They postulated that lack of influence on portal pressure might be associated with a decrease in portal venous inflow and a concomitant increase in portal-collateral resistance.[32] In addition to suppressing TNF-α, chronic thalidomide treatment obviously decreased plasma levels of VEGF. Fernandez et al demonstrated that anti-VEGF receptor-2 monoclonal antibody prevented collateral vessel formation without any significant effect on portal pressure in portal hypertensive mice.[36] They suggest that it was a reduction of the splanchnic blood flow and portal venous inflow due to decreased collateral angiogenesis by inhibition of VEGF. Chronic thalidomide treatment in cirrhotic rats had similar effects in decreasing plasma levels of VEGF without change of portal pressure in the current study. The more likely explanation is its potent effect in suppression of VEGF and decrease in portal venous inflow, which may partially account for the unchanged portal pressure in the thalidomide-treated cirrhotic rats.

In our study, long-term thalidomide administration improved vascular responsiveness to AVP in portal systemic collaterals at the concentration of 10^{-8} M in cirrhotic rats. It has been found that TNF-α plays a role in the mediation of vascular hyporesponsiveness in the mesenteric vessels of cirrhotic rats,[37] the aortic ring in rats with septic shock,[38] and persistent vasodilatation in the small intestinal microcirculation of rats with sepsis.[39] TNF-α might cause vasodilation through both prostaglandin and NO pathways, with the NO-dependent mechanism being the dominant one.[40,41]

The mechanism by which thalidomide treatment improves collateral vascular responsiveness to AVP may partially come from inhibition of TNF-α, though it did not reach statistical significance. However, the marked suppression of VEGF in chronic thalidomide-treated cirrhotic rats may be another important factor that influences vascular reactivity. VEGF is a potent activator of NO-dependent vasodilation that stimulates NO production by endothelial NO synthase and increases vascular permeability, and subsequently induces the migration and proliferation of endothelial cells, leading to expansion of pre-existing collateral vessels.[42] Chronic thalidomide treatment decreases plasma levels of VEGF, which may attenuate the production of NO, and then increases collateral vascular responsiveness to AVP in cirrhotic rats. Apart from suppression of VEGF, thalidomide and its analogs have also been demonstrated to inhibit expression of cyclooxygenase-2 from stimulated human peripheral blood monocytes.[43] In our study, thalidomide treatment did not elicit changes in EC50 values, suggesting that the receptor-mediated vasoconstriction effect is not influenced by thalidomide and the enhanced collateral vascular reactivity to AVP may be directed at the post-receptor level.

Although chronic thalidomide treatment produced a significant reduction in plasma VEGF levels and relatively lower TNF-α levels, the VEGF and TNF-α mRNA expressions of the left adrenal vein were not significantly altered after treatment. It has been shown that expressions of VEGF are upregulated in the jejunal mucosa microvasculature but not in the mesenteric vessels 24 hours after PVL.[44] However, Geerts et al demonstrated overexpression of VEGF in the mesentery microvasculature of portal hypertensive and cirrhotic rats.[45] The significant decrease in plasma VEGF levels without inhibition of VEGF mRNA expression in the left adrenal veins might be explained by the fact that thalidomide inhibits plasma VEGF levels via systemic inhibition of VEGF production with discrepant expressions of VEGF mRNA in different vascular territories. Another possible explanation is that thalidomide inhibits some kind of protease, such as eukaryotic initiation factor 4E (eIF-4E), without alteration in mRNA expression. eIF-4E is a crucial protease in VEGF production. Previous studies have demonstrated that enhanced VEGF expression is achieved through translational regulation rather than transcriptional regulation in cells with overexpression of eIF-4E.[46] VEGF is a glycoprotein that induces endothelial proliferation, angiogenesis and capillary hyperpermeabiliy.[47] Fernandez et al demonstrated that the formation of portal-systemic circulation might be due to angiogenesis driven by VEGF and modulated by VEGF receptor-2.[36,48] VEGF plays a crucial role in the portal hypertension-related collateral vessel formation that includes neoangiogenesis and reopening of pre-existing vessels. Thalidomide has been shown to inhibit the expression of VEGF, which is believed to be the mechanism for the attenuation of angiogenesis by thalidomide.[49]

To our knowledge, this is the first study to demonstrate that thalidomide can increase vascular reactivity to AVP in the portal-systemic collaterals of cirrhotic rats, partly related to decreased plasma VEGF levels. These findings suggest that the improved responsiveness to AVP occurs, at least in part, through VEGF downregulation after thalidomide administration in CBDL cirrhotic rats. The enhanced vasoconstrictive activities of AVP may implicate the synergistic effect to control acute gastroesophageal variceal bleeding. However, we should be cautious in extrapolating the results from animal models to humans and await further studies for elucidation.

Acknowledgments

This work was supported by grants from the National Science Council (NSC 93-2314-13-075-060) and Taipei Veterans General Hospital (VGH 94-223), Taiwan.

References

1. Garcia-Tsao G, Groszmann RJ, Fisher RL, Conn HO, Atterbury CE, Glickman M. Portal pressure, presence of gastroesophageal varices and variceal bleeding. *Hepatology* 1985;5: 419–24.
2. Tsai YT, Lay CS, Lai KH, Ng WW, Yeh YS, Chiang TT, Lee SD, et al. Controlled trial of vasopressin plus nitroglycerin vs. vasopressin alone in the treatment of bleeding esophageal varices. *Hepatology* 1986;6:406–9.
3. Gimson AES, Westaby D, Hegert J, Hegarty J, Watson A, Williams R. A randomized trial of vasopressin and vasopressin plus nitroglycerin in the control of acute variceal hemorrhage. *Hepatology* 1986;6:410–3.
4. Bosch J, Groszmann RJ, Garcia-Pagan JC, Teres J, Garcia-Tsao G, Navasa M, Mas A, et al. Association of transdermal nitroglycerin to vasopressin infusion in the treatment of variceal hemorrhage: a placebo controlled clinical trial. *Hepatology* 1989;10: 962–8.
5. Valla D, Girod C, Lee SS, Braillon A, Lebrec D. Lack of vasopressin action on splanchnic hemodynamics during bleeding: a study in conscious, portal hypertensive rats. *Hepatology* 1988;8:10–5.
6. Kravetz D, Bosch J, Teres J, Bruix J, Rimola A, Rodes J. Comparison of intravenous somatostatin and vasopressin infusions in treatment of acute variceal hemorrhage. *Hepatology* 1984;4:442–6.

7. Westaby D, Gimson A, Hayes PC, Williams R. Haemodynamic response to intravenous vasopressin and nitroglycerin in portal hypertension. *Gut* 1988;29:327–77.
8. Bosch J, Mastai R, Kravetz D, Bruix J, Rigau J, Rodés J. Measurement of azygos venous blood flow in the evaluation of portal hypertension in patients with cirrhosis. Clinical and hemodynamic correlations in 100 patients. *J Hepatol* 1985; 1:125–39.
9. Bosch J, Bordas JM, Mastai R, Kravetz D, Navasa M, Chesta J, Pizcueta MP, et al. Effects of vasopressin on the intravariceal pressure in patients with cirrhosis: comparison with the effects on portal pressure. *Hepatology* 1988;8:861–5.
10. Chan CC, Lee FY, Wang SS, Chang FY, Lin HC, Chu CJ, Tai CC, et al. Effects of vasopressin on portal-systemic collaterals in portal hypertensive rats: role of nitric oxide and prostaglandin. *Hepatology* 1999;30:630–5.
11. Chan CC, Wang SS, Lee FY, Chang FY, Lin HC, Hou MC, Huang HC, et al. Effects of vasopressin on portal-systemic collaterals of cirrhotic rats. *Scand J Gastroenterol* 2005;40:83–9.
12. Mellin GW, Katzenstein M. The saga of thalidomide: neuropathy to embriopathy, with case reports of congenital abnormalities. *N Engl J Med* 1962;267:1184–93.
13. Barnhill RL, McDougall AC. Thalidomide: use and possible mode of action in reaction lepromatous leprosy and in various other conditions. *Am Acad Dermatol J* 1982;7:317–23.
14. Londono F. Thalidomide in treatment of actinic prurigo. *Int J Dermatol* 1973;12:326–8.
15. Moreira AL, Sampaio EP, Zmuidzinas A, Frindt P, Smith KA, Kaplan G. Thalidomide exerts its inhibitory action on tumor necrosis factor-alpha by enhancing mRNA degradation. *J Exp Med* 1993;177:1675–80.
16. D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci USA* 1994; 91:4082–5.
17. Lopez-Talavera JC, Cadelina G, Olchowski J, Merril W, Groszmann RJ. Thalidomide inhibits tumor necrosis factors alpha, decreases nitric oxide synthesis, and ameliorates the hyperdynamic circulatory syndrome in portal-hypertensive rats. *Hepatology* 1996;23:1616–21.
18. Franco F, Gigou M, Szekely AM, Bismuth H. Portal hypertension after bile duct obstruction. Effect of the bile diversion on portal pressure in the rat. *Arch Surg* 1979;114:1064–7.
19. Cameron GR, Hasan SM. Disturbances of structure and function in the liver as the result of biliary obstruction. *J Pathol Bacteriol* 1958;75:33–49.
20. Kountouras J, Billing BH, Scheuer PJ. Prolonged bile duct ligation obstruction: a new experimental model of cirrhosis in the rat. *Br J Exp Pathol* 1984;65:305–11.
21. Lee FY, Colombato LA, Albillos A, Groszmann RJ. Administration of N^{ω}-nitro-L-arginine ameliorates portal-systemic shunting in portal-hypertensive rats. *Gastroenterology* 1993;105: 1464–70.
22. Mosca P, Lee FY, Kaumann AJ, Groszmann RJ. Pharmacology of portal-systemic collaterals in portal hypertensive rats: role of endothelium. *Am J Physiol* 1992;263:G544–50.
23. Hamilton RL, Berry MN, Williams MC, Severinghaus EM. A simple and inexpensive membrane "lung" for small organ perfusion. *J Lipid Res* 1974;15:182–6.
24. Chirgwin JM, Pryzbyla RJ, McDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979;18:5294–9.
25. Chamberlain LH, Buroyne RD. Identification of a novel cysteine string protein variant and expression of cysteine string proteins in non-neuronal cells. *J Biol Chem* 1996;271:7320–3.
26. Sampaio EP, Sarno EN, Galilly R, Cohn ZA, Kaplan G. Thalidomide selectively inhibits tumor necrosis factor alpha production by stimulated human monocytes. *J Exp Med* 1991; 173:699–703.
27. Keifer JA, Guttridge DC, Ashburner BP, Baldwin AS Jr. Inhibition of NF-kappa B activity by thalidomide through suppression of I kappa B kinase activity. *J Biol Chem* 2001;276: 22382–7.
28. Enomoto N, Takei Y, Hirose M, Ikejima K, Miwa H, Kitamura T, Sato N. Thalidomide prevents alcoholic liver injury in rats through suppression of Kupffer cell sensitization and TNF-alpha production. *Gastroenterology* 2002;123:291–300.
29. Tilg H, Wilmer A, Vogel W, Herold M, Nolchen B, Judmaier G, Huber C. Serum levels of cytokines in chronic liver diseases. *Gastroenterology* 1992;103:264–74.
30. Chu CJ, Lee FY, Wang SS, Lu RH, Tsai YT, Lin HC, Hou MC, et al. Hyperdynamic circulation of cirrhotic rats with ascites: role of endotoxin, tumour necrosis factor-alpha and nitric oxide. *Clin Sci* 1997;93:219–25.
31. Lopez-Talavera JC, Merrill WW, Groszmann RJ. Tumor necrosis factor α: a major contributor to the hyperdynamic circulation in prehepatic portal-hypertensive rats. *Gastroenterology* 1995; 108:761–7.
32. Muñoz J, Albillos A, Perez-Paramo M, Rossi I, Alvarez-Mon M. Factors mediating the hemodynamic effects of tumor necrosis factor-α in portal hypertensive rats. *Am J Physiol* 1999;276: G687–93.
33. Wang X, Han CD, Fiscus RR, Qi M, Jones SB. Hypotension- and endotoxin-induced alterations in calcitonin gene-related peptide: modulation by dexamethasone. *Circ Shock* 1991;34: 217–23.
34. Landry DW, Oliver JA. The ATP-sensitive K+channel mediates hypotension in endotoxinemia and hypoxic lactic acidosis in the dog. *J Clin Invest* 1992;89:2071–4.
35. Austin AS, Mahida YR, Clarke D, Ryder SD, Freeman JG. A pilot study to investigate the use of oxpentifylline (pentoxifylline) and thalidomide in portal hypertension secondary to alcoholic cirrhosis. *Aliment Pharmacol Ther* 2004;19: 79–88.
36. Fernandez M, Vizzutti F, Garcia-Pagan JC, Rodes J, Bosch J. Anti-VEGF receptor-2 monoclonal antibody prevents portal-systemic collateral vessel formation in portal hypertensive mice. *Gastroenterology* 2004;126:886–94.
37. Wiest R, Das S, Cadelina G, Garcia-Tsao G, Milstien S, Groszmann RJ. Bacterial translocation in cirrhotic rats stimulates eNOS-derived NO production and impairs mesenteric vascular contractility. *J Clin Invest* 1999;104:1223–33.
38. Takahashi K, Ando K, Ono A, Shimosawa T, Ogata E, Fujita T. Tumor necrosis factor-alpha induces vascular hyporesponsiveness in Sprague-Dawley rats. *Life Sci* 1992;50:1437–44.
39. Naziri W, Joshua IG. Role of tumor necrosis factor-alpha in small intestinal microcirculation. *Am Surg* 1998;64:203–9.
40. Baudry N, Vicaut E. Role of nitric oxide in effects of tumor necrosis factor-alpha on microcirculation in rat. *J Appl Physiol* 1993;75:2392–9.
41. Vicaut E, Hou X, Payen D, Bousseau A, Tedgui A. Acute effects of tumor necrosis factor on the microcirculation in rat cremaster muscle. *J Clin Invest* 1991;87:1537–40.
42. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003;9:669–76.
43. Payvandi F, Wu L, Haley M, Schafer PH, Zhang LH, Chen RS, Muller GW, et al. Immunomodulatory drugs inhibit expression of cyclooxygenase-2 from TNF-α, IL-1β, and LPS-stimulated human PBMC in a partially IL-10-dependent manner. *Cell Immunol* 2004;230:81–8.
44. Abraldes JG, Iwakiri Y, Loureiro-Silva M, Haq O, Sessa WC, Groszmann RJ. Mild increases in portal pressure upregulate vascular endothelial growth factor and endothelial nitric oxide

synthase in the intestinal microcirculatory bed, leading to a hyperdynamic state. *Am J Physiol Gastrointest Liver Physiol* 2006;290:G980–7.
45. Geerts AM, De Vriese AS, Vanheule E, Van Vlierberghe H, Mortier S, Cheung KJ, Demetter P, et al. Increased angiogenesis and permeability in the mesenteric microvasculature of rats with cirrhosis and portal hypertension: an *in vivo* study. *Liver Int* 2006;26:889–98.
46. Kevil CG, De Benedetti A, Payne DK, Coe LL, Laroux FS, Alexander JS. Translational regulation of vascular permeability factor by eukaryotic initiation factor 4E: implications for tumor angiogenesis. *Int J Cancer* 1996;65:785–90.
47. Thomas KA. Vascular endothelial growth factor, a potent and selective angiogenic agent. *J Biol Chem* 1996;271:603–6.
48. Fernandez M, Mejias M, Angermayr B, Garcia-Pagan JC, Rodes J, Bosch J. Inhibition of VEGF receptor-2 decreases the development of hyperdynamic splanchnic circulation and portal-systemic collateral vessels in portal hypertensive rats. *J Hepatol* 2005;43:98–103.
49. Gupta D, Treon SP, Shima Y, Hideshima T, Podar K, Tai YT, Lin B, et al. Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications. *Leukemia* 2001;15:1950–61.